The Plant Cell, Vol. 16, 1870–1882, July 2004, www.plantcell.org © 2004 American Society of Plant Biologists

Arabidopsis CAND1, an Unmodified CUL1-Interacting Protein, Is Involved in Multiple Developmental Pathways Controlled by Ubiquitin/Proteasome-Mediated Protein Degradation

Suhua Feng,[a] Yunping Shen,[a,b] James A. Sullivan,[a] Vicente Rubio,[a] Yue Xiong,[c] Tai-ping Sun,[d] and Xing Wang Deng[a,b,1]

[a] Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520-8104
[b] Peking-Yale Joint Center of Plant Molecular Genetics and Agrobiotechnology, College of Life Sciences, Peking University, Beijing 100871, China
[c] Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599
[d] Department of Biology, Duke University, Durham, North Carolina 27708-1000

Ubiquitin/proteasome-mediated protein degradation controls various developmental pathways in eukaryotes. Cullin-containing complexes are both versatile and abundant groups of RING family ubiquitin E3 ligases, whose activities are subject to control by RUB/Nedd8 (for related to ubiquitin/neural precursor cell-expressed developmentally downregulated 8) modification of their cullin subunits. Here, we report the identification of an *Arabidopsis thaliana* counterpart of human CAND1 (cullin-associated and neddylation-dissociated) and demonstrate that it can preferentially interact with unmodified CUL1. The Arabidopsis *cand1-1* null mutant displays distinct phenotypes, including late flowering, aerial rosettes, floral organ defects, low fertility, dwarfism, loss of apical dominance, and altered responses to multiple plant hormones. Molecular analyses show that many of these defects are because of compromised activity of CUL1-containing ubiquitin E3 ligases, indicating that CAND1 is required for their optimal activity. Furthermore, the *cand1-1* mutant displays a partial constitutive photomorphogenic phenotype and has defects in HY5 degradation in the absence of light, a process mediated by a different RING family E3, COP1. Thus, our data provides genetic support for a critical role of CAND1 in regulating various ubiquitin E3 ligases and their targeted cellular and developmental pathways.

INTRODUCTION

The ubiquitin/proteasome system is a universal selective proteolysis system in eukaryotes, in which target proteins are ubiquitinated and subsequently degraded by the 26S proteasome. Protein ubiquitination requires the coordinated action of a series of three distinct enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Hershko and Ciechanover, 1998). Ubiquitin E3 ligases are capable of recruiting substrates and catalyzing the transfer of ubiquitin moieties from the E2 to the substrates. Thus, they are largely responsible for the substrate specificity of the ubiquitin/proteasome system (Vierstra, 2003).

Cullin-containing complexes, which belong to the RING superfamily, are probably the most abundant group of ubiquitin E3 ligases. Cullin proteins can be clustered phylogenetically into five clades (Risseeuw et al., 2003). CUL1 is the best characterized cullin, which forms the SKP1/CUL1/ROC1/F-box protein (SCF) complex. SCF complexes can recruit ubiquitin-conjugated E2s through the RING finger protein ROC1 (also known as RBX1 or HRT1) and different substrates through divergent F-box proteins (Deshaies, 1999; N. Zheng et al., 2002). Some other cullin family members have also been shown to form SCF-like ubiquitin E3 ligase complexes in mammalian cells. CUL2 (or CUL5) forms a complex with ROC1, elongin B, elongin C, and BC-box proteins (Kamura et al., 1998, 2001; Kaelin, 2002). CUL3 forms a complex with the ROC1 and BTB proteins (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003b; Xu et al., 2003). CUL4A forms complexes with ROC1, DDB1, and DDB2, or CSA, or DET1/COP1 (Groisman et al., 2003; Wertz et al., 2004).

In *Arabidopsis thaliana*, although multiple cullins are present, only CUL1-containing SCF-type ubiquitin E3 ligases have been characterized at the biochemical and functional levels. On the other hand, Arabidopsis COP1, a repressor of photomorphogenesis in darkness, has been studied in detail. COP1 is a RING finger protein (von Arnim and Deng, 1993) and exhibits in vitro ubiquitin E3 ligase activity toward photomorphogenesis-promoting transcription factors on its own (Saijo et al., 2003; Seo et al., 2003), hence representing a different type of RING family E3. Recently, its human counterpart has been suggested to be part of a CUL4A-containing ubiquitin E3 ligase complex (Wertz et al., 2004).

The ubiquitin-like protein RUB (for related to ubiquitin; Arabidopsis and yeast), also known as Nedd8 (for neural precursor cell-expressed developmentally downregulated 8; human),

[1] To whom correspondence should be addressed. E-mail xingwang.deng@yale.edu; fax 203-432-5726.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Xing Wang Deng (xingwang.deng@yale.edu).
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.021949.

modifies all cullins examined so far. RUB/Nedd8 undergoes a ubiquitination-like E1/E2 cascade to form a covalent linkage with cullins, a process called rubylation or neddylation (Hochstrasser, 2000). RUB/Nedd8 modification stimulates the ubiquitin ligase activity of cullin-containing complexes (Furukawa et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000; del Pozo et al., 2002; Ohh et al., 2002). It has been suggested that RUB/Nedd8 can help ROC1 to recruit ubiquitin-conjugated E2s (Kawakami et al., 2001; N. Zheng et al., 2002). Interestingly, RUB/Nedd8-modified CUL1 is also the preferred form of CUL1 present in SCF complexes (Osaka et al., 2000; Read et al., 2000; Kawakami et al., 2001). Thus, the RUB/Nedd8 modification status of cullin plays a critical role in the assembly of cullin-containing ubiquitin E3 ligases.

Not surprisingly, RUB/Nedd8 deconjugation also plays an essential role in regulating cullin-containing ubiquitin E3 ligases. A highly conserved protein complex, the COP9 signalosome (CSN; Wei et al., 1998; Serino and Deng, 2003; Wei and Deng, 2003), has been shown to be capable of interacting with cullin-containing complexes and of cleaving RUB/Nedd8 off of the modified cullin (Lyapina et al., 2001; Schwechheimer et al., 2001; Zhou et al., 2001; Cope et al., 2002). Several groups have demonstrated that RUB/Nedd8 deconjugation negatively regulates the in vitro activity of cullin-containing ubiquitin E3 ligases (Lyapina et al., 2001; Zhou et al., 2001; Yang et al., 2002; Groisman et al., 2003). However, various genetic studies suggest that CSN is necessary for the optimal activity of multiple cullin-containing ubiquitin E3 ligases (Schwechheimer et al., 2001; Cope et al., 2002; Feng et al., 2003; Groisman et al., 2003; Liu et al., 2003; Pintard et al., 2003a; Wang et al., 2003). Therefore, it is hypothesized that dynamic cycles of RUB/Nedd8 conjugation and deconjugation are required to regulate assembly and activity of cullin-containing ubiquitin E3 ligases (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wolf et al., 2003). However, the mechanism for this regulation and the key players remain to be identified.

Recently, human CAND1 (cullin-associated and neddylation-dissociated), previously known as TIP120A (TATA binding protein interacting protein; Yogosawa et al., 1996), has been identified as a potential key player in the assembly of cullin-containing ubiquitin E3 ligases and has been shown to selectively bind unmodified cullins. Overexpressing CAND1 in cultured cell lines inhibits CUL1/SKP1 binding, and either RUB/Nedd8 modification or SKP1 binding dissociates CAND1 from CUL1 (Liu et al., 2002; J. Zheng et al., 2002; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003). Because CAND1 binding appears to antagonize RUB/Nedd8 modification, it is not surprising that in vitro cullin-containing ubiquitin E3 ligase activities are inhibited by CAND1 (Liu et al., 2002; J. Zheng et al., 2002; Min et al., 2003). Still, like CSN, CAND1 knockdown in cultured mammalian cells causes accumulation of SCF substrates, implying that CAND1 might be important for the optimal activity of cullin-containing ubiquitin E3 ligases (J. Zheng et al., 2002). However, because each of the mammalian studies was performed in vitro or in cultured cells, a validation by genetic analysis and revelation of CAND1's developmental functions will be essential.

In this article, we report a molecular genetic analysis of the Arabidopsis *CAND1* gene. We demonstrate that Arabidopsis CAND1 is preferentially associated with unmodified CUL1 in vivo. More importantly, we provide critical genetic evidence that CAND1 acts positively to regulate multiple ubiquitin E3 ligases and their associated developmental processes in plants.

RESULTS

Identification of the Arabidopsis *CAND1* Gene

A homology search using human *CAND1* sequence (Liu et al., 2002) identified a *CAND1* homolog, At2g02560, in the Arabidopsis genome. The presence of multiple EST clones as well as a full-length cDNA (RAFL09-95-I08) in the RIKEN collection supports the expression of At2g02560. The full-length open reading frame (ORF) of Arabidopsis *CAND1* was cloned by RT-PCR using RNA isolated from wild-type Arabidopsis seedlings, and sequence analysis confirmed its 100% identity to the reported full-length cDNA.

The Arabidopsis *CAND1* gene has 28 exons and encodes a protein of 1219 amino acids (accession number AY099857). The sequence identity of Arabidopsis CAND1 with other eukaryotic CAND1s is significant: 43% identity for mammals, 35% for *Drosophila melanogaster*, 33% for *Caenorhabditis elegans*, and 22% for fission yeast (*Schizosaccharomyces pombe*). A phylogenetic tree based on CAND1 protein sequence homologies shows that CAND1 is evolutionarily conserved (Figure 1A). In each of those organisms, the RUB/Nedd8 conjugation pathway is essential (Osaka et al., 2000; Tateishi et al., 2001; Kurz et al., 2002; Ou et al., 2002; Dharmasiri et al., 2003). Interestingly, CAND1 seems to be missing in budding yeast (*Saccharomyces cerevisiae*), where RUB/Nedd8 conjugation is not essential (Lammer et al., 1998; Liakopoulos et al., 1998). This is consistent with the notion that CAND1 may be functionally connected with RUB/Nedd8 modification.

Identification of *cand1* T-DNA Insertional Mutants

To investigate the developmental function of Arabidopsis CAND1, we took advantage of the superior genetics available in Arabidopsis. We searched the available Arabidopsis T-DNA insertional mutagenesis collections and obtained three independent lines (Figure 1B; Alonso et al., 2003; Rosso et al., 2003; see Methods). All three T-DNA insertional mutations studied behave recessively (data not shown). Polyclonal antibodies raised against Arabidopsis CAND1 can detect a single band migrating around 120 kD in wild-type Arabidopsis protein extracts but not in any of the three *cand1* homozygous T-DNA insertional alleles (Figure 1C). Thus, all three mutants likely represent true null or severe loss-of-function mutations for *CAND1*. Interestingly, the CUL1 protein level, in either the RUB-modified form or unmodified form, is not affected by any of the three *cand1* mutations (Figure 1C).

Initial Phenotype Analysis of *cand1* Mutants

All three *cand1* alleles produce similar phenotypes (Figure 1D). Basically, *cand1* affects multiple aspects of the plant phenotype throughout development. Germination and seedling stage growth of *cand1* mutants under normal conditions appear to be the same

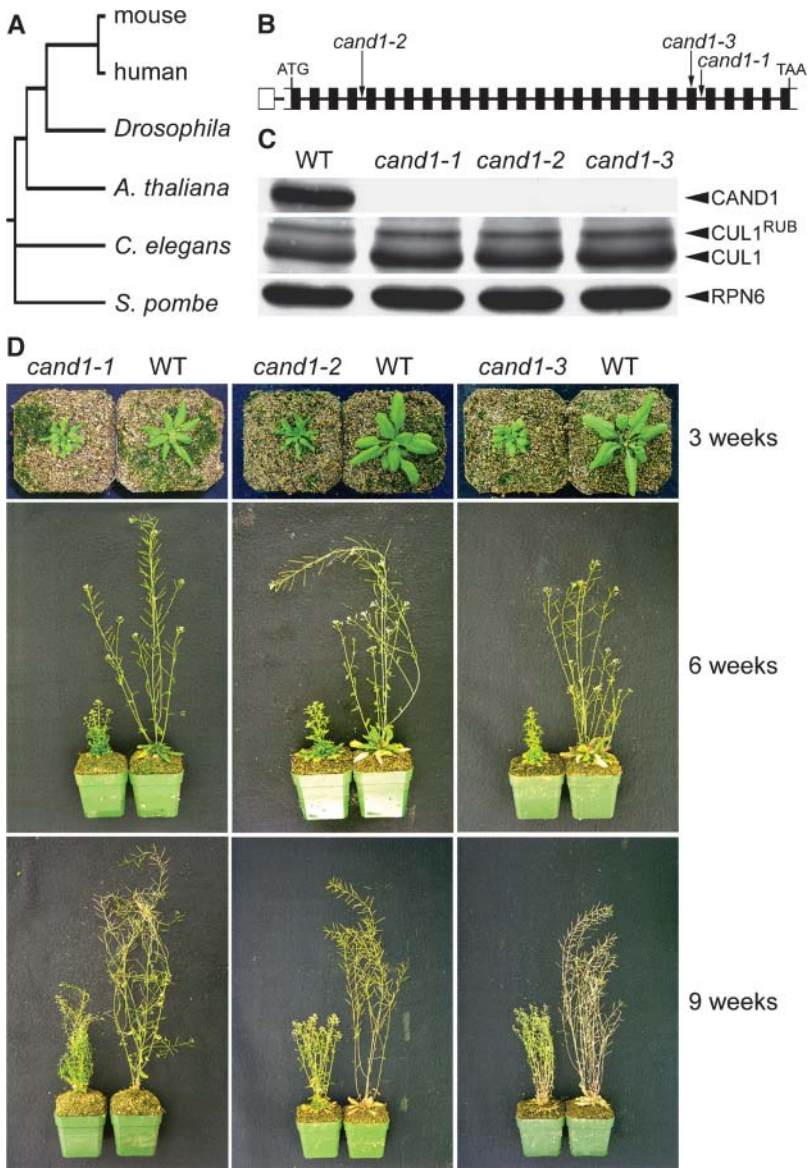

Figure 1. Identification and Characterization of Arabidopsis *CAND1* Gene and Its T-DNA Insertional Mutants.

(A) A phylogenetic tree of CAND1 proteins from representative eukaryotic organisms as labeled at the right.

(B) Schematic diagram of T-DNA insertions in the Arabidopsis *CAND1* gene (At2g02560). Exons are represented by closed (coding region) and open (untranslated regions) boxes, whereas introns are represented by lines. The T-DNA insertion sites of the three mutant alleles are indicated by arrows, with the assigned allele names for each insertional mutation at the top.

(C) The *cand1* mutations abolish CAND1 expression but do not affect CUL1 accumulation. Flower protein extracts were prepared from wild-type Arabidopsis and three *cand1* mutants and subjected to immunoblot analysis with anti-CAND1, anti-CUL1, and anti-RPN6 antibodies. Arrowheads indicate protein positions. RPN6 is used as a loading control.

(D) All three *cand1* mutants show similar phenotypes when grown under identical conditions. Numbers at the right of each row indicate the age of the plants when photographed.

as wild-type Arabidopsis (data not shown). The phenotypes of *cand1* mutants become more obvious later during development. As shown in Figure 1D, the rosette leaves of *cand1* mutants are much smaller than those of wild-type plants and have a wavy morphology. The *cand1* mutants flower later than wild-type plants, with an increased number of rosette leaves (Figures 1D and 4A), indicating that the vegetative to reproductive growth transition of the primary shoot apical meristem is affected. The growth of axillary meristems is also abnormal, where a large number of smaller leaves are produced (Figure 1D). Furthermore, the mutants have very low fertility, with less than one seed produced on average per silique (data not shown). Possibly

because of their poor fertility, mutant plants continue to make new flowers while wild-type plants of the same age start to senesce (Figure 1D).

Other notable phenotypes of *cand1* mutants include dwarfism and loss of apical dominance. When examined closely, dwarfism largely results from reduced stem elongation (Figure 1D; data not shown), which is analogous to the gibberellin pathway mutants (Harberd et al., 1998). The *cand1* mutants have a strong increase in the number of secondary inflorescences (Figure 1D), an indication that they have lost apical dominance, which is also observed in auxin response mutants (Lincoln et al., 1990; Estelle, 1992). In later sections, we will provide evidence that both gibberellin and auxin pathways are affected in the *cand1-1* mutant.

35S Promoter–Driven FLAG-CAND1 Partially Complements the *cand1-1* Mutation

To further confirm that the phenotypes observed are indeed attributable to *cand1* mutation, we constructed a chimeric gene using the constitutive 35S promoter of *Cauliflower mosaic virus* to drive expression of a *CAND1* cDNA, with three copies of flag tags at the N terminus of CAND1 protein. This construct was introduced into a *cand1-1* mutant background to test functional complementation. Because the homozygous *cand1-1* mutant produces very few seeds and is severely dwarfed, we first stably transformed the transgene into Arabidopsis plants heterozygous for *cand1-1* mutation. In T1 generation, we selected plants resistant to antibiotic markers for both the transgene and the T-DNA and further confirmed their genotypes through PCR-based genotyping and segregation in their selfed progenies (data not shown).

We examined expression levels of the FLAG-CAND1 fusion protein from the transgene as well as the endogenous CAND1 protein using total flower protein extracts. As shown in Figure 2A, although it's driven by a strong constitutive promoter, the level of FLAG-CAND1 protein is lower than endogenous CAND1 (lines 3-12 and 5-2). The FLAG-CAND1 expression is not enhanced in the *cand1-1* mutant background either (Figure 2A, lines 5-4 and 6-7). As a likely result of this low expression level of the FLAG-CAND1, *cand1-1* mutant phenotypes are only partially rescued by the transgene. Nevertheless, all of the key defects of *cand1* mutants discussed in the previous section, including late flowering time, low fertility, reduced plant size, loss of apical dominance, and other features, are partially rescued to various extents (Figure 2B, lines 5-4 and 6-7).

Furthermore, we identified several cosuppression lines, in which endogenous CAND1 protein abundance is downregulated by the presence of the transgene, and no FLAG-CAND1 fusion protein is expressed (Figure 2A, lines 5-22 and 6-2). Remarkably, like the partially rescued lines, the cosuppression lines also display intermediate phenotypes compared with wild-type and *cand1* mutants (Figure 2B). Thus, this observation supports the notion that FLAG-CAND1 is fully active and that the partial rescue in lines 5-4 and 6-7 is because of the low levels of the FLAG-CAND1 protein. Taken together, our data corroborates the linkage between the phenotypes and the *cand1* mutations. These

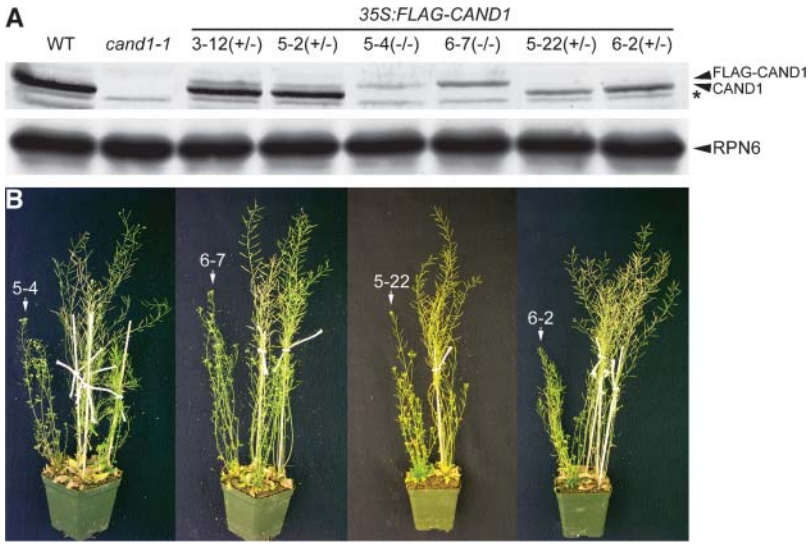

Figure 2. Complementation and Cosuppression Effect of the *35S:FLAG-CAND1* Transgene.

(A) Expression of the FLAG-CAND1 fusion protein. Flower protein extracts from wild-type Arabidopsis, *cand1-1* mutant, and various *35S:FLAG-CAND1* transgenic Arabidopsis lines (line numbers labeled at the top) were subjected to immunoblot analysis with anti-CAND1 and anti-RPN6 antibodies. Lines marked as +/− are heterozygous for *cand1-1* mutation, and lines marked as −/− are homozygous for the *cand1-1* mutation. Arrowheads indicate protein positions. The asterisk marks a cross-reacting band. RPN6 is used as a loading control.

(B) Comparison of partial complementation phenotypes and cosuppression phenotypes. An arrow is put at the top of each line with the name as labeled above. Plants shown next to the indicated lines are other transgenic Arabidopsis lines that are heterozygous for the *cand1-1* mutation. Pictures were taken when the plants were 7 weeks old.

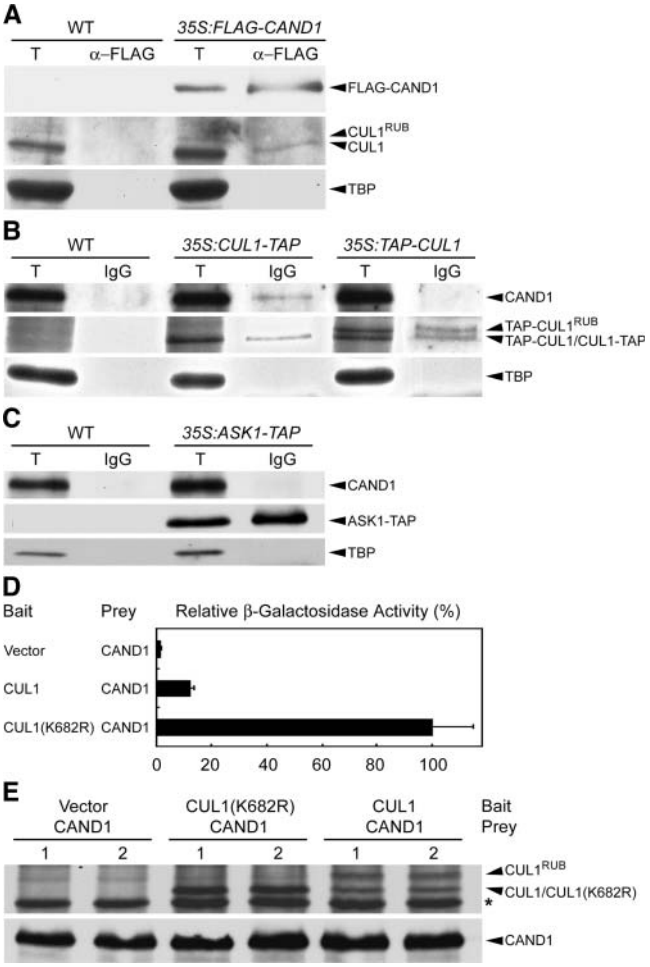

Figure 3. CAND1 Selectively Interacts with Unmodified CUL1 in Vivo.

(A) Unmodified CUL1 was precipitated together with FLAG-CAND1. Seedling protein extracts prepared from wild-type Arabidopsis and *35S:FLAG-CAND1* transgenic Arabidopsis (line 6-7) were incubated with anti-FLAG antibody conjugated agarose (α-FLAG). The precipitates and the total extracts were subjected to immunoblot analysis with antibodies against FLAG, CUL1, and TATA binding protein (TBP).

(B) and **(C)** CAND1 associates with CUL1-TAP but not with TAP-CUL1 or ASK1-TAP in vivo. Total flower protein extracts prepared from wild-type Arabidopsis, *35S:CUL1-TAP*, *35S:TAP-CUL1*, and *35S:ASK1-TAP* transgenic Arabidopsis were incubated with IgG-coupled sepharose. The precipitates and the total extracts were subjected to immunoblot analysis with antibodies against CAND1, TATA binding protein (TBP), and CUL1 **(B)** or MYC **(C)**.

In **(A)** to **(C)**, arrowheads indicate protein positions, and T indicates total protein extract. Anti-TBP (TATA binding protein) antibody is used as a pull-down control.

(D) CAND1 interacts with RUB modification site mutated CUL1 more strongly than with wild-type CUL1 in yeast two-hybrid assays. β-gal activity resulted from CUL1(K682R) and CAND1 interaction is set to 100%. Error bars represent standard deviation ($n = 4$).

(E) Arabidopsis CUL1 is modified by RUB in yeast, which can be abolished by a point mutation at its RUB modification site. Yeast strains used in two-hybrid assays (labeled at the top) were subjected to immunoblot analysis with antibodies against CUL1 and CAND1. Arrowheads indicate protein positions. The asterisk marks a nonspecific band cross-reacting with anti-CUL1 antibodies, which is also used as a loading control of the total protein amount in each lane.

results also indicate that the level of CAND1 is critically important, suggesting that CAND1 may act as a rate-limiting factor in some cullin-containing ubiquitin E3 ligase function.

CAND1 Preferentially Interacts with Unmodified CUL1 in Vivo

Because FLAG-CAND1 is functional, it was used in an initial attempt to detect in vivo association of CAND1 and CUL1 by immunoprecipitation. When we pulled down FLAG-CAND1 using anti-FLAG antibody in extracts from the transgenic seedlings, we were able to detect the presence of unmodified CUL1 in the precipitate (Figure 3A). This result suggests that, like in mammals, CAND1 interacts with unmodified CUL1 in Arabidopsis. Because of the low ratio of RUB-modified CUL1 to unmodified CUL1 in the transgenic seedlings, the low sensitivity of this coimmunoprecipitation does not allow a conclusion to be drawn as to whether the RUB-modified CUL1 was excluded from precipitation or whether it was simply below the detection threshold (Figure 3A).

Next, to determine the specificity of the CAND1/unmodified CUL1 interaction, we took advantage of wild-type Arabidopsis plants that express the CUL1 protein with a modified tandem affinity purification (TAP) tag fused at either the N or C terminus. Whereas nearly half of the TAP-CUL1 (N-terminal fusion) is modified by RUB, all of the CUL1-TAP (C-terminal fusion) exists in the unmodified form (Figure 3B). Because RUB modification takes place at the CUL1 C terminus, a big C-terminal fusion such as TAP (34 kD) presumably blocks RUB conjugation. On the other hand, an N-terminal TAP fusion has no spatial conflict with RUB conjugation and for some unknown reason slows down RUB deconjugation. The different properties of these two fusion proteins provide an ideal system for testing their differential associations with CAND1 in vivo. Indeed, CAND1 can be readily detected in the IgG precipitate of CUL1-TAP but not in TAP-CUL1 (Figure 3B). Additionally, consistent with observations in human cells (Liu et al., 2002; J. Zheng et al., 2002), CAND1 is not coimmunoprecipitated with ASK1-TAP (C-terminal fusion) in vivo (Figure 3C). This observation demonstrates that CAND1 does not interact with the C-terminal TAP tag itself, and the association between CAND1 and CUL1-TAP is specific.

To further characterize the interaction between Arabidopsis CAND1 and CUL1, we tested their interaction in a yeast two-hybrid assay. Wild-type CUL1 interacts with CAND1 in yeast, as shown by an eightfold increase in β-galactosidase (β-gal) activity over the vector control (Figure 3D). When the RUB modification site on CUL1 (del Pozo and Estelle, 1999) is mutated from Lys to Arg (K682R), the β-gal activity is further increased by 10-fold (Figure 3D). It is shown in Figure 3E that Arabidopsis CUL1 is indeed modified by RUB in yeast, whereas the mutant CUL1 protein is not. Moreover, it appears that the amount of the mutated CUL1(K682R) and the total amount of the modified and unmodified wild-type CUL1 are of similar levels (Figure 3E). Thus,

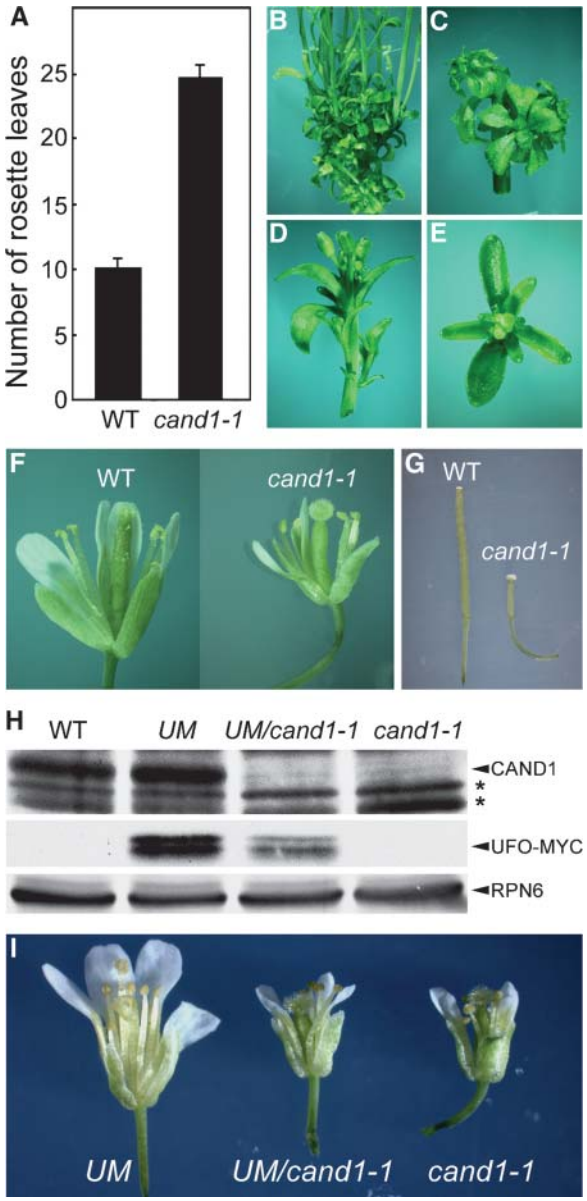

Figure 4. Abnormal Development of Shoot Apex and Flower in the *cand1-1* Mutant.

(A) Flowering time of wild-type Arabidopsis and *cand1-1* mutant, as indicated by average number of rosette leaves at the time of bolting. The identities of the plants are labeled at the bottom. Error bars represent standard deviation ($n = 6$).

(B) to **(E)** Aerial rosettes are formed in the axils of the *cand1-1* mutant. Samples were taken from different parts of the same plant, including the bottom **(B)**, the middle **(C)**, and the tip of the axillary branch (**[D]** and **[E]**).

(F) Comparison of wild-type and *cand1-1* flowers. The flowers were dissected to reveal the floral organs. Pictures were taken under the same magnification.

(G) Comparison of wild-type and *cand1-1* siliques.

(H) UFO-MYC protein level is greatly reduced in the *cand1-1* mutant background. Protein samples were extracted from flowers of wild-type Arabidopsis, *35S:UFO-MYC* transgenic Arabidopsis (*UM*), *cand1-1* mutant expressing *35S:FLAG-CAND1* transgene (*UM/cand1-1*), and *cand1-1* mutant. The extracts were then subjected to immunoblot analysis with anti-CAND1, anti-MYC, and anti-RPN6 antibodies. Arrowheads indicate protein positions. The asterisks mark two cross-reacting bands. RPN6 is used as a loading control.

(I) *UFO* overexpression phenotypes are overridden by *cand1-1* mutation. The identities of the flowers are indicated at the bottom. The flowers were dissected to reveal the floral organs.

the residual interaction observed between wild-type CUL1 and CAND1 could be because of the presence of a fraction of the wild-type CUL1 in the unmodified form in yeast. It is also interesting to note that in this yeast two-hybrid assay, the N terminus of CUL1 is fused to LexA (25 kD), which argues against the possibility that N-terminal TAP tag might prevent TAP-CUL1 and CAND1 from interacting with each other in vivo. Collectively, our studies show that CAND1 preferentially interacts with unmodified CUL1.

Loss of CAND1 Results in an Aberrant Vegetative-to-Reproductive Transition and Flower Development

As described earlier, *cand1* mutants flower later than wild-type plants, with an increased number of rosette leaves before bolting (Figures 1D and 4A). Even after they begin to flower, many more cauline leaves are still produced on the shoots, as compared with wild-type plants (Figure 1D). Furthermore, aerial rosettes are found in normal axillary branch positions (Figures 4B to 4E). The formation of aerial rosettes is attributable to the failure of axillary branch internodes to elongate immediately as they normally do in Columbia-0. This phenotype is observed in the late-flowering Arabidopsis ecotype Sy-0 (from Isle of Skye, UK), reflecting a defect in the vegetative/reproductive growth switch in the axillary meristem (Poduska et al., 2003).

The *cand1* mutant flowers have all the right organs, although they are much smaller (Figure 4F). We found that the mutant flower is poorly pollinated (data not shown). As a result, the silique of the mutant is very short and contains hardly any seeds (Figure 4G).

Arabidopsis *UFO* is required for the expression of B-class floral organ identity genes that control petal and stamen development and encodes an F-box protein that incorporates into an SCF-type ubiquitin E3 ligase (Samach et al., 1999; Wang et al., 2003). We have previously reported a *35S:UFO-MYC* overexpression line (*UM*), in which B-functions become ectopic and greatly enhanced, causing enlarged petal size, increased number of stamens, and filamentous carpels (Wang et al., 2003; Figure 4I). We introduced the *UFO-MYC* transgene into the *cand1-1* mutant background by genetic crossing. The *cand1-1* mutants expressing *UFO-MYC* were selected by protein gel blot analysis (Figure 4H), and their flower phenotypes were compared with *UM* and *cand1-1*. Essentially, the flowers of *UM/cand1-1* are identical to those of *cand1-1* mutant and drastically different from those of *UM* (Figure 4I). Consistent with the phenotype observation, the UFO-MYC protein level is greatly downregulated in *UM/cand1-1* flowers (Figure 4H). Evidently, SCF^{UFO} activity is suppressed in the *cand1-1* mutant, implying a positive regulatory role for

CAND1 in maintaining SCF^{UFO} ubiquitin E3 ligase activity during floral organ development.

The *cand1-1* Mutant Has Reduced Responsiveness to Jasmonate and Auxin

Another well-studied F-box protein in Arabidopsis is COI1, the central regulator of the jasmonate pathway (Xie et al., 1998; Xu et al., 2002). The *cand1-1* mutant shows a dosage-dependent resistance to the root growth inhibition effect caused by jasmonates, especially at low concentrations of jasmonates (2 and 5 μM), where *cand1-1* has an ~50% increase in resistance compared with the wild type (Figure 5A). The *cand1-1 coi1-1* double mutant has no enhanced resistance to jasmonates compared with the *coi1-1* single mutant (data not shown). This supports the idea that SCF^{COI1} controls most of the jasmonate-triggered responses (Feng et al., 2003) and that CAND1 acts to optimize its activity. However, unlike UFO-MYC, the COI1 protein level is not changed in the *cand1-1* mutant (data not shown).

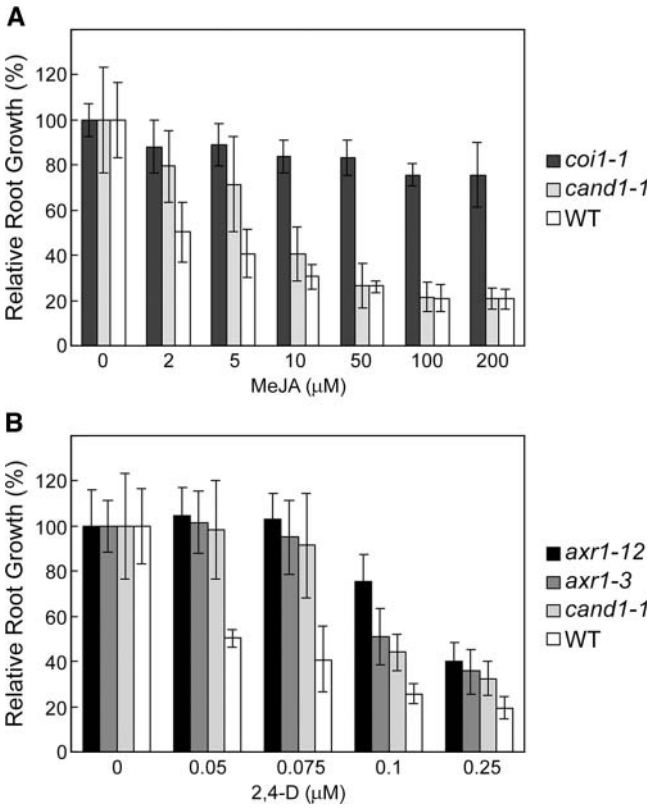

Figure 5. Jasmonate and Auxin Responses Are Affected in the *cand1-1* Mutant.

The root growth inhibitions conferred by jasmonate **(A)** and auxin **(B)** are reduced in the *cand1-1* mutant. Arabidopsis lines used in the experiments are indicated at the right. Concentrations of hormones used in MS medium are indicated at the bottom. Root length on MS medium without jasmonates or auxin was set as 100%, which did not show any significant difference among the wild type and various mutant lines. Error bars represent standard deviation ($n > 10$). MeJA, methyl jasmonate.

Thus, further evidence is required to substantiate that the enhanced resistance to jasmonates observed in *cand1-1* is because of the reduction in SCF^{COI1} activity.

The loss of apical dominance in the *cand1* mutants is reminiscent of some auxin pathway mutants. Indeed, we were able to demonstrate that root growth inhibition by auxin in *cand1-1* mutant is moderately relieved, which is comparable to *axr1-3*, a weak *axr1* mutant (Figure 5B). In an independent study, Chuang et al. (2004) identified a point mutation for Arabidopsis *CAND1*, which also causes an auxin-resistant phenotype and has synergistic effects with the *tir1-1* mutation. Like *UFO* and *COI1*, *TIR1* also encodes an F-box protein, and SCF^{TIR1} is required for auxin responses (Gray et al., 1999). Together, these data provide evidence that optimal SCF^{TIR1} activity requires CAND1 function.

CAND1 Is Required for Proper Gibberellin Signaling

As mentioned earlier, the dwarf phenotype of *cand1* mutants might be caused by defects in gibberellin pathways. To test this hypothesis, we examined whether loss of CAND1 impairs gibberellin (GA) signaling pathways that also involve a specific SCF-type ubiquitin E3 ligase. SCF^{SLY1} is suggested to be responsible for targeting the GA pathway repressor of ga1-3 (RGA) for degradation in Arabidopsis (McGinnis et al., 2003). Indeed, whereas RGA protein in wild-type Arabidopsis is barely visible in protein gel blot analysis, *sly1-10*, a mutant of the F-box protein in SCF^{SLY1}, can accumulate a high level of RGA (McGinnis et al., 2003; Figure 6A). Noticeably, the RGA protein level is also elevated in the *cand1-1* mutant (Figure 6A), which likely contributes to the mutant dwarf phenotype.

RGA protein is destabilized rapidly upon addition of GA (Silverstone et al., 2001; McGinnis et al., 2003; Figure 6B, lanes 1 and 2). However, in the *cand1-1* mutant, the GA-induced RGA protein reduction is partially compromised (Figure 6B, lanes 3 and 4). Therefore, it is conceivable that CAND1 is a positive regulator of SCF^{SLY1} and that in *cand1* mutants, SCF^{SLY1} cannot achieve optimal activity, which results in a reduction of GA signaling.

It is interesting to note that GA responses regulate floral pathway integrators (Simpson and Dean, 2002), which could provide a possible explanation for the late flowering phenotype of *cand1* mutants. In addition, GA is also essential for petal and stamen development in Arabidopsis (Harberd et al., 1998), indicating another potential cause of the *cand1* mutant flower phenotypes.

CAND1 Represses Photomorphogenesis by Promoting HY5 Degradation in Darkness

As stated earlier, loss of CAND1 does not affect germination and seedling stage growth under normal conditions. However, when *cand1-1* seedlings are grown in darkness, they exhibit mild constitutive photomorphogenic phenotypes, with short hypocotyls and opened cotyledons (Figure 7A). It has been shown that the extent of photomorphogenic development is directly correlated with the abundance of HY5, a photomorphogenesis-promoting transcription factor (Osterlund et al., 2000).

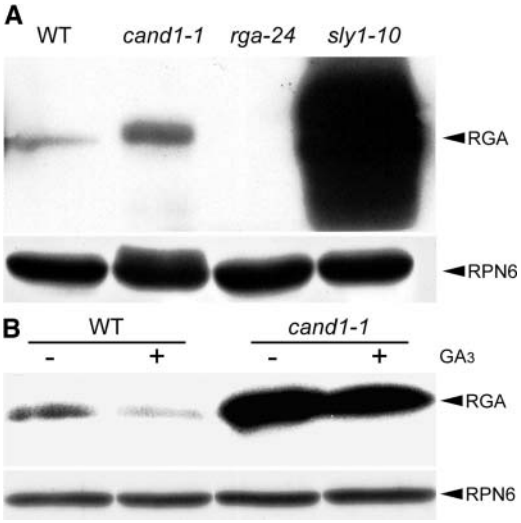

Figure 6. Proper GA Signaling Requires CAND1.

(A) The GA pathway negative regulator RGA accumulates in the *cand1-1* mutant. Flower protein extracts from wild-type Arabidopsis, *cand1-1*, *rga-24* (negative control), and *sly1-10* mutants were subjected to immunoblot analysis with anti-RGA and anti-RPN6 antibodies.

(B) The *cand1-1* mutant fails to rapidly destabilize RGA upon GA treatment. Eight-day-old wild-type and *cand1-1* seedlings were treated with GA_3 for 2 h. Subsequently, protein extracts were prepared from treated (+) and untreated (−) seedlings and subjected to immunoblot analysis with anti-RGA and anti-RPN6 antibodies.

The arrowheads in **(A)** and **(B)** indicate protein positions. RPN6 is used as a loading control.

Consistent with this, we found that dark-grown *cand1-1* mutant seedlings, whose degree of photomorphogenesis is in between light-grown and dark-grown wild-type seedlings, also accumulate an intermediate level of HY5 protein (Figure 7C, lanes 1 to 3). HY5 is degraded by the 26S proteasome upon transfer from light to dark (Osterlund et al., 2000; Figure 7D, lanes 1 to 3). Evidently, HY5 protein is more stable in the *cand1-1* mutant (Figure 7D, lanes 4 to 6).

The targeted degradation of HY5 in darkness requires COP1, a ubiquitin E3 ligase for HY5 (Saijo et al., 2003), and COP10, an E2 ubiquitin-conjugating enzyme variant (Suzuki et al., 2002). We crossed the *cand1-1* mutant with *cop1-6* and *cop10-4*, both of which are nonlethal alleles, to obtain genetic evidence for the functional interactions of CAND1 with COP1 and COP10. Remarkably, the dark-grown double mutants *cop1-6 cand1-1* and *cop10-4 cand1-1* have shorter hypocotyls than their parental single mutants (Figure 7B) and accumulate higher levels of HY5 as well (Figure 7C, lanes 4 to 7). These data suggest that CAND1 has synergistic effects with COP1 and COP10 in promoting HY5 degradation and in repressing photomorphogenesis in darkness. Considering that human COP1 has most recently been indicated to be an integral part of a CUL4A-containing ubiquitin E3 ligase (Wertz et al., 2004), it will be interesting to find out if this function of CAND1 is dependent on Arabidopsis CUL4.

DISCUSSION

In this study, we characterized Arabidopsis CAND1, a crucial player in the regulation of cullin-containing ubiquitin E3 ligases. We demonstrated that CAND1 has the ability to selectively interact with unmodified CUL1 in vivo. The Arabidopsis *cand1-1* null mutant exhibits serious defects in various aspects of development, and we showed that many of these defects are caused by compromised activities of CUL1-containing SCF-type ubiquitin E3 ligases. Therefore, CAND1 functions as a positive

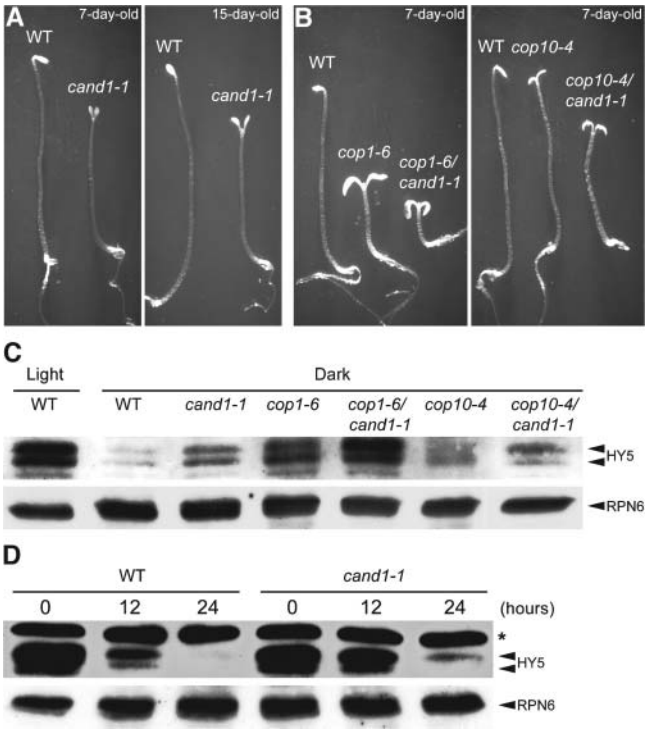

Figure 7. CAND1 Functions in Photomorphogenesis by Regulating HY5 Degradation.

(A) and **(B)** Loss of CAND1 leads to constitutive photomorphogenesis in dark and enhances phenotypes of weak *cop* alleles. Different Arabidopsis lines (labeled at the top) were grown in complete darkness for the indicated number of days. All pictures were taken under the same magnification.

(C) The *cand1-1* mutation causes hyperaccumulation of HY5 in dark-grown seedlings. Seedling protein extracts from 4-d-old light-grown wild-type Arabidopsis, dark-grown wild-type Arabidopsis, and various dark-grown single mutants and double mutants (labeled at the top) were prepared and blotted by anti-HY5 and anti-RPN6 antibodies.

(D) HY5 is degraded less efficiently in the *cand1-1* mutant than in wild-type Arabidopsis. Four-day-old light-grown seedlings of wild-type Arabidopsis and *cand1-1* mutant were transferred to complete darkness. Samples were collected at different time points starting from the transfer (indicated at the top) and blotted with anti-HY5 and anti-RPN6 antibodies.

The arrowheads and asterisk in **(C)** and **(D)** indicate protein positions and a cross-reacting band, respectively. HY5 has two forms: unphosphorylated (bottom band) and phosphorylated (top band). RPN6 is used as a loading control.

regulator of cullin-containing E3s. In addition, we found that CAND1 also works together with COP1 and COP10 to promote HY5 degradation, which sheds new light on the control of photomorphogenesis.

Physical Interaction between CAND1 and CUL1

Here, we provide three lines of direct in vivo evidence supporting that Arabidopsis CAND1 is an unmodified CUL1-interacting protein, similar to its human counterpart (Liu et al., 2002; J. Zheng et al., 2002; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003). First, only the unmodified form of CUL1 is detected in the immunoprecipitate of FLAG-CAND1 (Figure 3A). Second, CAND1 can be pulled down together with the unmodified CUL1-TAP; contrarily, TAP-CUL1, which is hyper-RUB modified, does not pull down any CAND1 (Figure 3B). Third, when the RUB modification site on Arabidopsis CUL1 is mutated and its RUB modification in yeast is prevented (Figure 3E), the interaction between Arabidopsis CUL1 and CAND1 in yeast increases by 10-fold (Figure 3D). It is interesting to note that in yeast approximately half of the wild-type CUL1 actually exists in the unmodified form, yet its ability to interact with CAND1 decreases ~10-fold compared with CUL1(K682R) mutant protein, despite the fact that the two CUL1 proteins have almost equal abundance (Figures 3D and 3E). In Arabidopsis, TAP-CUL1 has a high rate of RUB modification in comparison with CUL1-TAP (Figure 3B). If this causes a similar scale of decrease in the interaction between the unmodified form of TAP-CUL1 and CAND1, the interaction might fall below the detection limit of our IgG pull-down experiment (Figure 3B).

The observation mentioned above can be taken as an indication that the interaction between CUL1 and CAND1 may be labile and dynamic in vivo. What is consistently observed in both Arabidopsis and yeast is that if CUL1 is not able to undergo RUB modification or if its capacity for RUB modification is reduced, its interaction with CAND1 is strong. On the contrary, when CUL1 is hyper-RUB modified or with a high capacity for RUB modification, the interaction between CUL1 and CAND1 decreases dramatically, to an extent much below expectation based on the abundance of the remaining CUL1 in the unmodified form (Figure 3). Taken together, it suggests that the binding of CAND1 to unmodified CUL1 is quickly turned over by RUB modification, and only when RUB modification cannot take place, CAND1 binding to CUL1 will be sustained.

In vivo, when a target protein somehow signals a need for degradation, the substrate/SKP1/F-box protein subcomplex promotes RUB/Nedd8 modification of CUL1, which leads to CAND1 dissociation from CUL1. Reciprocally, when RUB/Nedd8 is cleaved from modified CUL1 by CSN, CAND1 binds unmodified CUL1 with high affinity, displacing the SKP1/F-box protein. Therefore, through CAND1, the RUB/Nedd8 conjugation and deconjugation cycle can be directly linked to the assembly/disassembly of cullin-containing ubiquitin E3 ligases. This model implies that although CAND1 binding is an important step in the RUB/Nedd8 conjugation and deconjugation cycles, it may not affect the RUB/Nedd8 modification status of cullins, which is supported by our data (Figure 1C).

Regulation of SCF-Type Ubiquitin E3 Ligases by CAND1

The unique binding characteristic of CAND1 turns out to be the key to its biological function. It prevents the formation of functional cullin-containing ubiquitin E3 ligases by sequestering ROC1/cullin. Consistently, various in vitro assays show that CAND1 acts as a negative regulator of SCF ubiquitin E3 ligase activities (Liu et al., 2002; J. Zheng et al., 2002; Min et al., 2003). However, CAND1 binding of unmodified cullins should be considered as an intermediate step in the cullin-containing complex dynamic assembly/disassembly cycles; hence, CAND1 is an integral component in the overall ubiquitin/proteasome-mediated degradation of protein substrates (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wolf et al., 2003). This would suggest a positive role for CAND1 in ubiquitin E3 ligase–mediated developmental processes.

We revealed the physiological and developmental roles of CAND1 in Arabidopsis through examination of T-DNA insertional mutants. Multiple SCF-type ubiquitin E3 ligases are likely to be compromised in the *cand1-1* mutant, including SCF^{UFO}, SCF^{TIR1}, SCF^{COI1}, and SCF^{SLY1}. SCF^{UFO} promotes the expression of AP3 and PI, the B-class floral organ identity genes (Samach et al., 1999). Flower phenotypes of *UFO* overexpression are overridden by *cand1-1* mutation, which suggests that SCF^{UFO} requires CAND1 for its optimal activity (Figure 4I). SCF^{COI1} and SCF^{TIR1} control jasmonate and auxin pathways, respectively (Gray et al., 1999; Xu et al., 2002). Loss of CAND1 seems to affect their activities, because the *cand1-1* mutant has reduced responses to both jasmonate and auxin (Figure 5). Reduction of SCF^{TIR1} activity is also indicated by the loss of apical dominance in *cand1* mutants (Figure 1D). SCF^{SLY1} targets the GA pathway repressor RGA for degradation, stimulating the GA response (McGinnis et al., 2003). The *cand1-1* mutant accumulates a higher level of RGA protein than the wild type (Figure 6A) and fails to rapidly degrade RGA upon GA treatment (Figure 6B). Adult *cand1* mutant plants are dwarfed, with severely reduced stem elongation (Figure 1D). This evidence suggests that CAND1 is necessary for normal SCF^{SLY1} function and proper GA response. Collectively, our genetic studies have undoubtedly established CAND1 as a positive regulator of CUL1-containing SCF-type ubiquitin E3 ligases.

It is clear that RUB/Nedd8 conjugation and deconjugation cycles are important for many CUL1-containing SCF-type ubiquitin E3 ligases because loss or reduction of RUB/Nedd8 modification, RUB/Nedd8 cleavage, or absence of CAND1 will impair their activities. Why is dynamic assembly/disassembly important for the activity of cullin-containing E3? One theory is that F-box proteins assembled in the SCF complexes are subjected to the E3's self-ubiquitination activity (Zhou and Howley, 1998; Wirbelauer et al., 2000; Li et al., 2004). By limiting their incorporation into the complexes, they can be stabilized and work more efficiently when needed (J. Zheng et al., 2002; Cope and Deshaies, 2003; Wei and Deng, 2003). In the *cand1-1* mutant, we observed a drastic decrease of UFO-MYC overexpression level (Figure 4H), but the endogenous COI1 level is not changed (data not shown). Obviously, there is no simple answer to this question.

The nonlethal nature and relative absence of a phenotypic defect during embryonic and seedling development of the null *cand1* mutant suggested that CAND1 is not required for all SCF-type ubiquitin E3 ligases because the *cul1* null mutant itself is embryonic lethal (Shen et al., 2002). Also, considering the fact that defects in RUB conjugation (Dharmasiri et al., 2003) or deconjugation (Serino and Deng, 2003; Wei and Deng, 2003) cause severe seedling growth retardation and adult lethality, CAND1 may not be an absolutely essential component in mediating RUB/Nedd8 conjugation and deconjugation cycles of all cullin-containing ubiquitin E3 ligases.

Control of Flowering and Photomorphogenesis by CAND1

We successfully related several *cand1* mutant phenotypes to the activities of known CUL1-containing SCF-type ubiquitin E3 ligases. There are other abnormities of *cand1* mutants that cannot be readily explained in this way, for example, the late flowering phenotype and aerial rosettes (Figures 1D and 4A to 4E). Reduction of SCF-type ubiquitin E3 ligase activities in *cand1* mutants may play a part in this because GA pathway mutants, as well as mutants for the two F-box proteins ZTL and FKF, which are involved in circadian clock control, are late flowering. Both GA and circadian clock pathways act upstream of floral pathway integrators that regulate flowering time (Nelson et al., 2000; Somers et al., 2000; Simpson and Dean, 2002). However, mutations in these pathways alone do not cause formation of aerial rosettes. Another example is the flower phenotype (Figures 4F and 4G), which apparently cannot be attributed to the inactivity of any single cullin-containing ubiquitin E3 ligase known in Arabidopsis. A quick explanation is that a combination of defects in multiple ubiquitin E3 ligase activities eventually causes *cand1* phenotypes, keeping in mind that there are at least 694 putative F-box proteins in the Arabidopsis genome for CUL1-containing SCF-type ubiquitin E3 ligases (Gagne et al., 2002) and that there are many other cullins yet to be examined. In addition, we cannot eliminate a role for cullin-independent CAND1 function in flower timing and flower development.

Photomorphogenesis is repressed in darkness through the proteasomal degradation of photomorphogenesis-promoting transcription factors, such as HY5 (Osterlund et al., 2000). In this pathway, the E3 is COP1, a different RING family ubiquitin E3 ligase (Saijo et al., 2003; Seo et al., 2003). COP10, a ubiquitin-conjugating enzyme variant, is also necessary for HY5 degradation (Suzuki et al., 2002). Surprisingly, CAND1 has a role in promoting HY5 degradation, as shown by the high level of HY5 in the dark-grown constitutive photomorphogenic *cand1-1* mutant (Figures 7A and 7C), and in the failure of the *cand1-1* mutant to rapidly destabilize HY5 upon transfer from light to dark (Figure 7D). Furthermore, CAND1 has synergistic genetic interactions with both COP1 and COP10 (Figures 7B and 7C), supporting its direct involvement in repressing photomorphogenesis. Interestingly, human COP1 has recently been shown to assemble into a CUL4A-containing ubiquitin E3 ligase for c-Jun (Wertz et al., 2004), and Arabidopsis COP1 does form complexes in vivo (Saijo et al., 2003). Therefore, it is reasonable to speculate that CAND1's regulation of photomorphogenesis is realized by modulating an orthologous ubiquitin E3 ligase in Arabidopsis that contains COP1 and CUL4 and targets photomorphogenesis-promoting transcription factors. Alternatively, it is possible that another cullin-containing ubiquitin E3 ligase may be indirectly involved in regulating COP1 E3 activity.

METHODS

Plant Materials, Growth Conditions, and Hormone Treatments

The *axr1-3*, *axr1-12* (Lincoln et al., 1990), *coi1-1* (Xie et al., 1998), *rga-24* (Silverstone et al., 1998), *sly1-10* (McGinnis et al., 2003), *cop1-6* (McNellis et al., 1994), and *cop10-4* (Suzuki et al., 2002) mutants and the *35S*:*UM* (Wang et al., 2003) transgenic plants were described previously. The wild-type *Arabidopsis thaliana* plants used in this study were of the Columbia-0 ecotype.

To grow Arabidopsis seedlings, seeds were surface sterilized, put on MS plates (Gibco, Cleveland, OH) containing 1% sucrose, and cold treated at 4°C for 3 to 5 d before being placed in a standard, continuous white light growth chamber or in complete darkness at 22°C. To obtain adult plants, 7- to 9-d-old light-grown seedlings were transferred to soil and grown in a standard long-day (16 h light/ 8 h dark) growth room.

For root growth inhibition assays, Arabidopsis seedlings were first grown on normal MS medium for 4 d and then transferred to MS medium containing different concentrations of methyl jasmonate (Bedoukian, Danbury, CT) or 2,4-D (Sigma, St. Louis, MO). Root length was measured 4 d after the transfer. GA treatment experiments were performed as previously described (Silverstone et al., 2001).

Cloning of Arabidopsis *CAND1* and Isolation of *cand1* T-DNA Insertional Mutants

Full-length ORF of *CAND1* was amplified by RT-PCR from wild-type Arabidopsis seedlings with forward primer (5′-CGCGGATCCGCATGGCGAACTTACAAGTTTC-3′) and reverse primer (5′-ATAAGAATGCGGCCGCTTACTCATTCCGGATTGTC-3′). The *Bam*HI/*Not*I fragment of the PCR product was cloned into pEG202 (Origene, Rockville, MD) and then sequenced. This construct is named as pEG-CAND1, which served as the PCR template for subsequent cloning of *CAND1* into other vectors.

Based on a database search, we found one T-DNA insertion line for *CAND1* in the GABI-Kat collection (Line ID 134H10; Rosso et al., 2003). Plants homozygous for the T-DNA insertion were identified by PCR-based genotyping. This insertional mutant is named *cand1-1*. The mutation cosegregates with the T-DNA insertion through a backcross to wild-type plants. Two additional T-DNA insertional mutants, *cand1-2* and *cand1-3*, were found in the Salk collection (from Salk_099479 and Salk_110969, respectively; Alonso et al., 2003). The mutant plants were identified within segregating populations by phenotype resemblance to the *cand1-1* mutant and confirmed by PCR-based genotyping. When not specified, we use *cand1* to indicate all three mutants throughout the text.

Generation of Transgenic Arabidopsis Plants

A *Kpn*I/*Sal*I fragment containing full-length *CAND1* ORF was inserted into pF3PZPY122 (Feng et al., 2003). Then, an *Xba*I/*Sal*I fragment with the inserted DNA was subcloned into pJIM19(BAR), a plant binary vector that has basta-resistance marker and the 35S promoter of *Cauliflower mosaic virus*.

Arabidopsis plants heterozygous for *cand1-1* mutation were used in the transformation of *35S*:*FLAG-CAND1* transgene. Transgenic plants were selected with sulfadiazine (100 μg/mL; Sigma) and gulfosinate-ammonium (20 μg/mL; Riedel-de Haën, Seelze, Germany). The *cand1-1* mutants carrying *35S*:*FLAG-CAND1* transgene were identified by PCR-based genotyping.

Full-length ORFs of Arabidopsis *CUL1* and *ASK1* were cloned via Gateway reactions (Invitrogen, Carlsbad, CA) into plant binary vector with either N-terminal or C-terminal modified TAP tag fusion (Saijo et al., 2003). The transgenes were individually introduced into wild-type Arabidopsis. Transgenic plants were selected with gentamicin (200 μg/mL; Sigma).

Immunoblot Assays and Antibodies

Arabidopsis tissues were homogenized in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor (Roche, Indianapolis, IN). The extracts were centrifuged twice at 4°C for 10 min each, and the protein concentration in the supernatant was determined by Bradford assay (Bio-Rad, Hercules, CA). Protein samples were boiled in sample buffer, run on SDS-PAGE gels (8, 12, or 17.5%), and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were probed with different primary antibodies.

A *Bam*HI/*Not*I fragment containing the first 630 nucleotides of *CAND1* ORF was cloned into pET-28a (Novagen, Madison, WI). This construct encodes a fusion protein with 6X His tags and CAND1's N-terminal 210 residues. The fusion protein was expressed in *Escherichia coli* and purified with nickel-nitrilotriacetic acid agarose beads (Qiagen, Valencia, CA). Polyclonal antibodies were raised by immunizing rabbits using purified fusion protein as antigen.

Other primary antibodies used in this study include anti-CUL1 (Wang et al., 2002), anti-RGA (Silverstone et al., 2001), anti-HY5 (Osterlund et al., 2000), anti-COI1 (Xu et al., 2002), anti-RPN6 (Kwok et al., 1999), anti-TBP (Schwechheimer et al., 2001), anti-MYC (Saijo et al., 2003), and anti-FLAG (Sigma).

Yeast Two-Hybrid and in Vivo Pull-Down Analyses

Procedures for yeast two-hybrid assays and bait construct for CUL1 have been described previously (Schwechheimer et al., 2001; Wang et al., 2002). To create a CUL1(K682R) construct, the adenosine at position 2045 of *CUL1* ORF was mutated by PCR method to guanosine. A *Sal*I fragment containing full-length *CAND1* ORF was cloned into pJG4/5 (Origene) to make the CAND1 prey construct. For protein expression level detection, yeast cells were boiled directly in sample buffer and subjected to immunoblot analysis.

The FLAG antibody pull-down and IgG pull-down experiments were performed as previously described (Feng et al., 2003; Saijo et al., 2003) with minor modifications. The recipe of lysis/binding/washing buffer was 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 25 mM β-glycerolphosphate, 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor (Roche). The acid elution and concentration steps were omitted.

Sequence data for the protein sequences used in the phylogenetic analysis have been deposited with the EMBL/GenBank data libraries under accession numbers XP_125901 (mouse), NP_060918 (human), NP_609389 (Drosophila), NP_178360 (Arabidopsis), NP_507244 (*C. elegans*), and NP_593286 (*S. pombe*). The Arabidopsis *CAND1* gene has been deposited under the accession number AY099857.

ACKNOWLEDGMENTS

We are grateful to Y. Liu and S.P. Dinesh-Kumar for the TAP-fusion plant binary vector; to T. Tsuge for the monoclonal MYC antibodies; to P. Figueroa for the HY5 antibodies; to N. Wei for the *35S:UFO-MYC* plants; to J.G. Turner for the *coi1-1* mutant; to M. Estelle for the *axr1-3* and *axr1-12* mutants; to C.M. Steber for the *sly1-10* mutant; to J. Habashi and C. McCall for critical reading of the manuscript; and to B. Gray for communicating results prior to publication. The *cand1-1* was generated in the context of the GABI-Kat program and provided by B. Weisshaar (MPI for Plant Breeding Research; Cologne, Germany). We also thank Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants. This research was supported by grants from National Science Foundation (MCB-0077217 and NSF 2010 MCB-0115870) to X.W.D. J.A.S. and V.R. are supported by long-term fellowships from the Human Frontier Science Program.

Received February 19, 2004; accepted April 30, 2004.

REFERENCES

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301,** 653–657.

Chuang, H., Zhang, W., and Gray, W.M. (2004). Arabidopsis *ETA2*, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF^{TIR1} ubiquitin ligase. Plant Cell **16,** 1883–1897.

Cope, G.A., and Deshaies, R.J. (2003). COP9 signalosome: A multifunctional regulator of SCF and other cullin-based ubiquitin ligases. Cell **114,** 663–671.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., and Deshaies, R.J. (2002). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science **298,** 608–611.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. **15,** 435–467.

Dharmasiri, S., Dharmasiri, N., Hellmann, H., and Estelle, M. (2003). The RUB/Nedd8 conjugation pathway is required for early development in *Arabidopsis*. EMBO J. **22,** 1762–1770.

Estelle, M. (1992). The plant hormone auxin: Insight in sight. Bioessays **14,** 439–444.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003). The COP9 signalosome interacts physically with SCF^{COI1} and modulates jasmonate responses. Plant Cell **15,** 1083–1094.

Furukawa, M., He, Y.J., Borchers, C., and Xiong, Y. (2003). Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. Nat. Cell Biol. **5,** 1001–1007.

Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (2000). The CUL1 C-terminal sequence and ROC1 are required for nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. Mol. Cell. Biol. **20,** 8185–8197.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., and Vierstra, R.D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **99,** 11519–11524.

Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D.A. (2003). BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. Mol. Cell **12,** 783–790.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev. **13,** 1678–1691.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is

differentially regulated by the COP9 signalosome in response to DNA damage. Cell **113,** 357–367.

Harberd, N.P., King, K.E., Carol, P., Cowling, R.J., Peng, J., and Richards, D.E. (1998). Gibberellin: Inhibitor of an inhibitor of...? Bioessays **20,** 1001–1008.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. **67,** 425–479.

Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. **2,** E153–E157.

Hwang, J.W., Min, K.W., Tamura, T.A., and Yoon, J.B. (2003). TIP120A associates with unneddylated cullin 1 and regulates its neddylation. FEBS Lett. **541,** 102–108.

Kaelin, W.G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. Nat. Rev. Cancer **2,** 673–682.

Kamura, T., Burian, D., Yan, Q., Schmidt, S.L., Lane, W.S., Querido, E., Branton, P.E., Shilatifard, A., Conaway, R.C., and Conaway, J.W. (2001). Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase. J. Biol. Chem. **276,** 29748–29753.

Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W.G., Jr., Conaway, R.C., and Conaway, J.W. (1998). The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes Dev. **12,** 3872–3881.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. EMBO J. **20,** 4003–4012.

Kurz, T., Pintard, L., Willis, J.H., Hamill, D.R., Gonczy, P., Peter, M., and Bowerman, B. (2002). Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. Science **295,** 1294–1298.

Kwok, S.F., Staub, J.M., and Deng, X.W. (1999). Characterization of two subunits of *Arabidopsis* 19S proteasome regulatory complex and its possible interaction with the COP9 complex. J. Mol. Biol. **285,** 85–95.

Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCF^{Cdc4} complex. Genes Dev. **12,** 914–926.

Li, Y., Gazdoiu, S., Pan, Z.Q., and Fuchs, S.Y. (2004). Stability of homologue of Slimb F-box protein is regulated by availability of its substrate. J. Biol. Chem. **279,** 11074–11080.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. EMBO J. **17,** 2208–2214.

Lincoln, C., Britton, J.H., and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell **2,** 1071–1080.

Liu, C., Powell, K.A., Mundt, K., Wu, L., Carr, A.M., and Caspari, T. (2003). Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms. Genes Dev. **17,** 1130–1140.

Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002). NEDD8 modification of CUL1 dissociates $p120^{CAND1}$, an inhibitor of CUL1-SKP1 binding and SCF ligases. Mol. Cell **10,** 1511–1518.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (2001). Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. Science **292,** 1382–1385.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P., and Steber, C.M. (2003). The Arabidopsis *SLEEPY1* gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. Plant Cell **15,** 1120–1130.

McNellis, T.W., von Arnim, A.G., Araki, T., Komeda, Y., Misera, S., and Deng, X.W. (1994). Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. Plant Cell **6,** 487–500.

Min, K.W., Hwang, J.W., Lee, J.S., Park, Y., Tamura, T.A., and Yoon, J.B. (2003). TIP120A associates with cullins and modulates ubiquitin ligase activity. J. Biol. Chem. **278,** 15905–15910.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). *FKF1*, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. Cell **101,** 331–340.

Ohh, M., Kim, W.Y., Moslehi, J.J., Chen, Y., Chau, V., Read, M.A., and Kaelin, W.G., Jr. (2002). An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells. EMBO Rep. **3,** 177–182.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh-E, A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. EMBO J. **19,** 3475–3484.

Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., and Nakayama, K.I. (2003). Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. Biochem. Biophys. Res. Commun. **303,** 1209–1216.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. Nature **405,** 462–466.

Ou, C.Y., Lin, Y.F., Chen, Y.J., and Chien, C.T. (2002). Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in *Drosophila* eye development. Genes Dev. **16,** 2403–2414.

Pintard, L., Kurz, T., Glaser, S., Willis, J.H., Peter, M., and Bowerman, B. (2003a). Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in *C. elegans*. Curr. Biol. **13,** 911–921.

Pintard, L., Willis, J.H., Willems, A., Johnson, J.L., Srayko, M., Kurz, T., Glaser, S., Mains, P.E., Tyers, M., Bowerman, B., and Peter, M. (2003b). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. Nature **425,** 311–316.

Poduska, B., Humphrey, T., Redweik, A., and Grbic, V. (2003). The synergistic activation of *FLOWERING LOCUS C* by *FRIGIDA* and a new flowering gene *AERIAL ROSETTE 1* underlies a novel morphology in Arabidopsis. Genetics **163,** 1457–1465.

Podust, V.N., Brownell, J.E., Gladysheva, T.B., Luo, R.S., Wang, C., Coggins, M.B., Pierce, J.W., Lightcap, E.S., and Chau, V. (2000). A Nedd8 conjugation pathway is essential for proteolytic targeting of $p27^{KIP1}$ by ubiquitination. Proc. Natl. Acad. Sci. USA **97,** 4579–4584.

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M. (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis cullin AtCUL1 is required for auxin response. Plant Cell **14,** 421–433.

del Pozo, J.C., and Estelle, M. (1999). The *Arabidopsis* cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. Proc. Natl. Acad. Sci. USA **96,** 15342–15347.

Read, M.A., Brownell, J.E., Gladysheva, T.B., Hottelet, M., Parent, L.A., Coggins, M.B., Pierce, J.W., Podust, V.N., Luo, R.S., Chau, V., and Palombella, V.J. (2000). Nedd8 modification of Cul-1 activates $SCF^{\beta TrCP}$-dependent ubiquitination of $I\kappa B\alpha$. Mol. Cell. Biol. **20,** 2326–2333.

Risseeuw, E.P., Daskalchuk, T.E., Banks, T.W., Liu, E., Cotelesage, J., Hellmann, H., Estelle, M., Somers, D.E., and Crosby, W.L. (2003). Protein interaction analysis of SCF ubiquitin E3 ligase subunits from *Arabidopsis*. Plant J. **346,** 753–767.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized

population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. **53,** 247–259.

Saijo, Y., Sullivan, J.A., Wang, H., Yang, J., Shen, Y., Rubio, V., Ma, L., Hoecker, U., and Deng, X.W. (2003). The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev. **17,** 2642–2647.

Samach, A., Klenz, J.E., Kohalmi, S.E., Risseeuw, E., Haughn, G.W., and Crosby, W.L. (1999). The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. Plant J. **20,** 433–445.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (2001). Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TIR1} in mediating auxin response. Science **292,** 1379–1382.

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L., and Chua, N.H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature **424,** 995–999.

Serino, G., and Deng, X.W. (2003). The COP9 signalosome: Regulating plant development through the control of proteolysis. Annu. Rev. Plant Biol. **54,** 165–182.

Shen, W.H., Parmentier, Y., Hellmann, H., Lechner, E., Dong, A., Masson, J., Granier, F., Lepiniec, L., Estelle, M., and Genschik, P. (2002). Null mutation of *AtCUL1* causes arrest in early embryogenesis in *Arabidopsis*. Mol. Biol. Cell **13,** 1916–1928.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T. (1998). The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell **10,** 155–169.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T.P. (2001). Repressing a repressor: Gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. Plant Cell **13,** 1555–1566.

Simpson, G.G., and Dean, C. (2002). *Arabidopsis*, the Rosetta stone of flowering time? Science **296,** 285–289.

Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.A. (2000). *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. Cell **101,** 319–329.

Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (2002). *Arabidopsis* COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. Genes Dev. **16,** 554–559.

Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001). The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. J. Cell Biol. **155,** 571–579.

Vierstra, R.D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. **8,** 135–142.

von Arnim, A.G., and Deng, X.W. (1993). Ring finger motif of *Arabidopsis thaliana* COP1 defines a new class of zinc-binding domain. J. Biol. Chem. **268,** 19626–19631.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W., and Wei, N. (2003). The COP9 signalosome interacts with SCF^{UFO} and participates in Arabidopsis flower development. Plant Cell **15,** 1071–1082.

Wang, X., Kang, D., Feng, S., Serino, G., Schwechheimer, C., and Wei, N. (2002). CSN1 N-terminal-dependent activity is required for Arabidopsis development but not for Rub1/Nedd8 deconjugation of cullins: A structure-function study of CSN1 subunit of COP9 signalosome. Mol. Biol. Cell **13,** 646–655.

Wei, N., and Deng, X.W. (2003). The COP9 signalosome. Annu. Rev. Cell Dev. Biol. **19,** 261–286.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X.W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Curr. Biol. **8,** 919–922.

Wertz, I.E., O'Rourke, K.M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R.J., and Dixit, V.M. (2004). Human De-Etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. Science **303,** 1371–1374.

Wirbelauer, C., Sutterluty, H., Blondel, M., Gstaiger, M., Peter, M., Reymond, F., and Krek, W. (2000). The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: Evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J. **19,** 5362–5375.

Wolf, D.A., Zhou, C., and Wee, S. (2003). The COP9 signalosome: An assembly and maintenance platform for cullin ubiquitin ligases? Nat. Cell Biol. **5,** 1029–1033.

Wu, K., Chen, A., and Pan, Z.Q. (2000). Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. J. Biol. Chem. **275,** 32317–32324.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). *COI1*: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280,** 1091–1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14,** 1919–1935.

Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T.H., Vidal, M., Elledge, S.J., and Harper, J.W. (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature **425,** 316–321.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Xiao, D., Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (2002). The COP9 signalosome inhibits $p27^{KIP1}$ degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. Curr. Biol. **12,** 667–672.

Yogosawa, S., Makino, Y., Yoshida, T., Kishimoto, T., Muramatsu, M., and Tamura, T. (1996). Molecular cloning of a novel 120-kDa TBP-interacting protein. Biochem. Biophys. Res. Commun. **229,** 612–617.

Zheng, N., et al. (2002). Structure of the Cul1-Rbx1-Skp1-F box^{Skp2} SCF ubiquitin ligase complex. Nature **416,** 703–709.

Zheng, J., Yang, X., Harrell, J.M., Ryzhikov, S., Shim, E.H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002). CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. Mol. Cell **10,** 1519–1526.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R., and Wolf, D.A. (2001). The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. BMC Biochem. **2,** 7–17.

Zhou, P., and Howley, P.M. (1998). Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Mol. Cell **2,** 571–580.